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Research Article

Evaluation of Antibacterial and Anti-oxidant Activity of Some Lichens of Uttarakhand

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Abstract:

Objective: The present study was undertaken to investigate antimicrobial and antioxidant activity of ethanolic extract of Parmelia sexatilis, Parmelia caperata, Parmelia parletum, Everniastrum cirrhatum, Parmelia pereoridisum, Parmoterma mesotropum, Parmoterma reticulatum, Parmoterma perlatum, and Parmelia squarrosa collected from different locations of Uttarakhand.

Methods: Antimicrobial activity was assayed using disc diffusion and two-fold dilution method. Free radical scavenging potential was assayed using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and total phenol content equivalent to gallic acid.

Results: All the species of lichen were effective against the *Staphylococcus aureus*, out of them 7 were effective against *Mycobacterium smegmatis* and *Micrococcus luteus*, and 6 against *Bacillus cereus*. The extracts of *Parmelia caperata* (8-32 µg/ml MIC), *Parmelia parletum* (16-32 µg/ml MIC), *Parmelia sexatilis* (16-32 µg/ml MIC) and *Everniastrum cirrhatum* (8-32 µg/ml MIC) showed the strongest antibacterial activity. The extract did not show activity against fungi. Most of the lichen species were effective against *M. smegmatis* and *B. cereus*. It was also found that the tested extracts exhibited the highest radical scavenging activity with the greatest amount of phenolic content. The highest value of phenols was seen in the ethanol extract of *Parmelia caperata* at a 162.02 µg of gallic acid equivalent while *Everniastrum cirrhatum* extracts showed 160 µg of gallic acid equivalent. The scavenging effects of all lichen extract range from 17.44 – 84.33.7%. Extracts from lichen *Parmelia sexatilis*, and *Parmelia caperata* showed largest DPPH radical scavenging activity (84.33%) which was similar or greater than the standard antioxidants, ascorbic acid (86.5%), BHA (79.78%) and a-tocopherol (63.99%).

Conclusion: Lichens are the potent source of antimicrobial and anti-oxidant metabolites. Further studies are required so that the potent lichen species under the study may serve as leads in the development of potential antibacterial and antioxidant agents.

Keywords: Lichens; antimicrobial activity; anti-oxidant activity; free radical

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Introduction

Lichens are complex plants living in the symbiotic relationship with fungi and algae [1] and have been used traditionally as medicine for treatment of various diseases [2]. These commercially used as spices, dyes, foods, medicines, animal feed, architect models, wreath and floral decorations, perfumes, and as test organisms for atmospheric pollution [3-5]. The metabolites of lichens had a wide variety of biological actions, including antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects [1]. They synthesize and produce numerous compounds, "lichen substances'', including aliphatic, cycloaliphatic, aromatic and terpenic components [6] which have a distinguished antimicrobial activity [7-8]. Keeping these facts in mind, the researchers have focused on lichens to find out some new chemical entity from lichens as, there is a continuous emergence of drug resistance among the major bacterial pathogens. Therefore, there is a need to develop more effective drugs to combat these pathogens. In this context, the present study was undertaken to screen some lichens of Uttarakhand for antibacterial activity.

2. Materials and Methods

2.1 Collection of samples

The Lichens were collected from Garhwal region of Uttrakhand, India in March 2013. Their identity was confirmed, and voucher specimens were deposited in the Institute's herbarium. The plants used in the experiment, and their voucher numbers are given in Table 1.

Table 1 Lichens taken in the study

Voucher Number	Identified Lichen species	Family	Growth Form	Sustrate	Place of Collection
MBB01	***Parmelia sexatilis***	Parmeliaceae	Foliose	Plant Bark	Mussoorie
MBB02	***Parmelia caperata***	Parmeliaceae	Foliose	Plant Bark	Dehradun
MBB03	***Parmelia parletum***	Parmeliaceae	Foliose	Plant Bark	Dehradun
MBB04	***Everniastrum cirrhatum***	Parmeliaceae	Foliose	Plant Bark	Mussoorie
MBB05	***Parmelia pereoridisum***	Parmeliaceae	Foliose	Plant Bark	Ranichori
MBB06	***Parmoterma mesotropum***	Parmeliaceae	Foliose	Plant Bark	Ranichori
MBB07	***Parmoterma reticulatum***	Parmeliaceae	Foliose	Plant Bark	Ranichori
MBB08	***Parmoterma perlatum***	Parmeliaceae	Foliose	Plant Bark	Dehradun
MBB09	***Parmelia squarrosa***	Parmeliaceae	Foliose	Plant Bark	Ranichori

2.2 Test Microorganisms

The bacterial cultures, *Staphylococcus aureus* MTCC96, *Micrococcus luteus* MTCC106, *Bacillus subtilis* MTCC441, *Escherichia coli*

MTCC2939, *Pseudomonas aeruginosa* MTCC424, and *Mycobacterium smegmatis* MTCC6 and the fungal pathogens such as *Candida albicans* MTCC1637, *Microsporum gypseum* MTCC2829, *Trichophyton rubrum* MTCC296 were procured from Microbial Type Culture Collection (MTCC), Chandigarh, India while clinical isolates of *Proteus vulgaris*, *Enterococcus fecalis E. coli* from Departmental Culture Collection, Department of Microbiology, Sardar Bhagwan Singh PG institute of Biomedical Sciences and Research, Balawala, Dehradun, India.

2.3 Preparation of lichen Extract

The lichen extract was prepared according to previously described methodology [9]. The extraction was repeated two times. The ethanol was removed by evaporation using rotavapour (Buchi, Flawil, Switzerland) under pressure (175 mbar) and temperature (50 ℃). The ethanolic extracts were kept at room temperature (28–30 ℃) for drying. The crude extracts were prepared by dissolving known amount of the dry extracts in dimethyl sulfoxide (DMSO; Merck, Goa, India), to have a stock solution of 100 mg/L) concentration. The final concentration of DMSO did not extend 2%.

2.4 Preliminary screening for their antibacterial activity

Preliminary screening of lichen extract was performed using well diffusion method. The overnight bacterial cultures were spread on the plates of Muller Hinton agar and wells of 6 mm diameter were made in the centre of the plates with the help of cork borer. 50 µL of the test samples (100 µg/well final conc.) were transferred into the wells and plates were incubated at 37 ℃ for 24 h. All the experiments were repeated twice and data presented were the average of their independent readings.

2.5 Determination of MIC and MBC values of lichen samples by broth dilution method

The MIC of the test samples was determined by twofold micro-dilution method using sterile flat-bottom 96-well polystyrene micro-titre plates (Axygen, CA, USA). The test samples were diluted serially twofold with nutrient broth and the culture-inoculated microtitre plates were incubated at 37 ℃ for 48 h. The growth was recorded spectrophotometrically at 600 nm. The MIC is the lowest concentration of a drug that prevents the growth of a particular pathogen. On the other hand, the minimal lethal or bactericidal concentration is the lowest drug that kills the pathogen.

2.6 Determination of Total Phenol Content (TPC) by Folin-Ciocalteau method

Total phenol content of developed formulations was determined by Folin-Ciocalteau method [10]. Different concentrations of gallic acid were used to draw the standard graph and total phenol content of the sample was expressed as equivalent to miligram gallic acid per ml of solution [11].

2.7 Radical Scavenging Activity by DPPH◦ method

Free-radical scavenging activity against 2, 2-diphenyl-1-picrylhydrazyl (DPPH◦) radical was evaluated following the method of

Bermudez-Soto & Tomas-Barberan [11] and Surveswaran *et al*. [12]. The DPPH radical (DPPH◦) solution (0.004 %) was prepared in 80 % ethanol [13]. Different concentrations of samples (0.05, 0.1, 0.15 and 0.2 g/mL) were prepared for the study. The DPPH◦ solution (3.9 ml) was added to 0.1 ml of the samples. The reaction for scavenging DPPH◦ was carried out at room temperature in the dark for 1h and then the reduction in absorbance was recorded at 515 nm. The reaction was followed with a PerkinElmer LAMBDA-25 UV-VIS spectrophotometer. All the measurements were performed in triplicate.

The percentage decrease of the absorbance at 515 nm was calculated by the formula as below [14].

$$I = [(A_B - A_A) / A_B] \text{ X } 100$$

Where I = DPPH◦ inhibition%, AB = Absorbance of the blank sample (t = 0 min); AA = absorbance of the test sample at the end of the reaction (t = 60 min).

Statistical analysis

Mean and standard deviation was calculated using Microsoft Excel 2007 program. The graphs were prepared using MicrocalTM Origin version 6.0. In figure, error bars indicate standard error of the mean, where error bars are not visible; they are smaller than the marker.

3. Results

The collected samples of lichens were identified by Botanical Survey of India Dehradun, Uttrakhand (Table 1). The crude powder of samples were obtained after vacuum drying and packed in vial and stored at 4 ℃ for further use.

3.1 Antibacterial activity

All the lichen species tested in the present study were active against the *Staphylococcus aureus* while 7 were effective against *Mycobacterium smegmatis* and *Micrococcus luteus*, and 6 against *Bacillus cereus*. None of the species were found effective against *Bacillus subtilis*, *Proteus vulgaris*, *Pseudomonas aerugenosa*, *Staphylococcus saprophyticus*, *Enterococcus fecalis*, and *Escherichia coli* and fungi (therefore not mentioned). The antimicrobial activities of different lichens are given in Table 2. As clear from the Table 2 that most of the lichen showed antibacterial activity against *B. cereus*, *Mycobacterium smegmatis*, and *Micrococcus luteus* and *S. aureus* while *Everniastrum cirrhatum* and *Parmelia pereoridisum* showed activity against *P. aeruginosa*. The lichen that showed most promising antibacterial activity was taken for further study and MIC and MBC values were determined.

The ethanolic extracts of *Parmelia sexatilis* were found to be active with MIC of 16 µg/mL against *B. cereus*, while *Mycobacterium smegmatis*, *Staphylococcus aureus*, *Micrococcus luteus*, showed higher MIC values. The MBC value was observed at higher concentration except in case of S. aureus where MIC was equal to MBC (Table 3).

The ethanolic extracts of *Parmelia caperata* were found to be active with a MIC of 8 µg/mL against *S. aureus* while 16 µg/mL against *M. smegmatis* and *B. cereus*. Higher MIC value was recorded with M. luteus (Table 3). Moreover, higher MBC values

were also recorded as compared to MIC value (Table 3).

The ethanolic extracts of *Parmelia parletum* were found to be active with a MIC of 16 µg/mL against *S.aureus*, *M. smegmatis*, and *M. luteus*. Higher MIC value was recorded with *B. cereus* (Table 3).Moreover, higher MBC values were also recorded as compared to MIC value except with *S. aureus*, in which MBC value was recorded same as that of MIC value (Table 3).

Table 2 Antibacterial activity of Lichens against test organisms

Test Organisms	**Inhibition Zone Diameter in mm*** (Sample concentration: 100 µg/well)**								
	Sample 1	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**	**Sample 6**	**Sample 7**	**Sample 8**	**Sample 9**
E. coli	-	-	-	-	-	-	-	-	-
B. cereus	25.00± 1.00	27.00± 1.00	23.00± 3.00	21.00± 0.00	18.00± 0.00	21.00± 2.00	15.00± 0.00	17.00± 0.00	16.00± 2.00
M. smegmatis	27.00± 2.00	32.00± 1.00	28.00± 1.00	26.00± 1.00	19.00± 0.00	17.00± 1.00	16.00± 0.00	18.00± 2.00	18.00± 1.00
M. luteus	20.00± 1.00	12.00± 2.00	20.00± 0.00	25.00± 0.00	-	-	21.00± 2.00	14.00± 1.00	19.00± 0.00
B. subtilis	-	-	-	-	-	-	-	-	-
P. vulgaris	-	-	-	-	-	-	-	-	-
P. aerugenosa	-	-	-	06.00± 1.00	05.00± 0.00	-	-	-	-
E. fecalis	-	-	-	-	-	-	-	-	-
S. aureus	18.00± 1.00	22.00± 2.00	18.00± 0.00	20.00± 0.00	10.00± 2.00	12.00± 1.00	16.00± 1.00	12.00± 0.00	10.00± 0.00
S. saprophyticus	-	-	-	-	-	-	-	-	-
Candida albicans	-	-	-	-	-	-	-	-	-
Microsporum Gypseum	-	-	-	-	-	-	-	-	-
Trichophyton rubrum	-	-	-	-	-	-	-	-	-

*Average of triplicates ± standard deviation; ** The final concentration of DMSO was 2% and did not showed antimicrobial activity (negative control); 1, Parmelia sexatilis; 2, Parmelia caperata; 3, Parmelia

parletum; 4, Everniastrum cirrhatum; 5, Parmelia pereoridisum; 6, Parmoterma mesotropum; 7, Parmoterma reticulatum; 8, Parmoterma perlatum; 9, Parmelia squarrosa

Table 3 MIC* and MBC* value of potential lichens against test organisms

Microorganisms	*Parmelias exatilis*		*Parmelia caperata*		*Parmelia parletum*		*Everniastrum cirrhatum*	
	MIC (µl/ml)	MBC (µl/ml)	MIC (µl/ml)	MBC (µl/ml)	MIC (µl/ml)	MBC (µl/ml)	MIC (µl/ml)	MBC (µl/ml)
Mycobacterium smegmatis	32	64	16	32	16	32	64	64
Bacillus cereus	16	32	16	64	32	64	8	32
Staphylococcus aureus	32	32	8	16	16	16	32	64
Micrococcus luteus	32	64	32	64	16	32	16	32

*Final concentration of DMSO did not extend 2% and did not showed antimicrobial activity; values are mean of triplicates.

The ethanolic extracts of *Everniastrum cirrhatum* were found to be active with a MIC of 8, 16, 32 and 64 µg/mL against *B. cereus*, *M. luteus*, *S.aureus*, and *M. smegmatis* respectively. Moreover, higher MBC values were also recorded as compared to MIC value except with *M. smegmatis*, in which MBC value was recorded same as that of MIC value (Table 3).

3.2 Determination of total phenolic content

Total phenolic contents of tested extracts are given in fig. 1. The amount of total phenolic compounds was determined as equivalent to gallic acid. Highest phenolic compound was identified in extract of *Parmelia caperata* at a 162.02 µg of gallic acid equivalent while *Everniastrum cirrhatum* extracts showed 160 µg of gallic acid equivalent. Other lichens, *Parmelia sexatilis*, *Parmelia parletum*, *Parmelia pereoridisum*, *Parmoterma mesotropum*, and *Parmoterma reticulatum*, showed total phenolic content of 111.11, 100, 86.33, 62.47, 84.27 µg of gallic acid equivalent while *Parmoterma perlatum*, and *Parmelia squarrosa* showed lowest phenolic content (Fig.1)

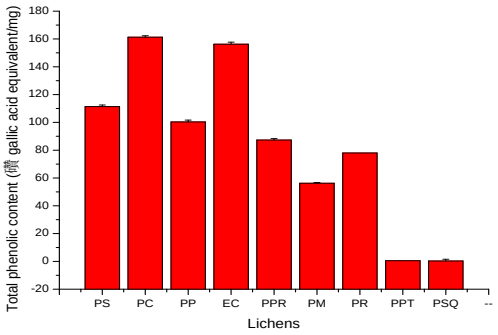

Figure 1 Total phenolic content of lichens; PS, Parmelia sexatilis; PC, Parmelia caperata; PP, Parmelia parletum; EC, Everniastrum cirrhatum; PP, Parmelia pereoridisum; PM, Parmoterma mesotropum; PR, Parmoterma reticulatum; PPT, Parmoterma perlatum; PSQ, Parmelia squarrosa, bar indicates the value of standard deviation

3.3 Radical Scavenging Activity by DPPH method

The scavenging DPPH radicals of the studied lichen extracts is shown in Figure 2. Ethanolic extracts of the tested lichens showed a good scavenging activity on DPPH radical. The scavenging effects of all lichen extract range from 17.44-84.33.7%. Extracts from lichen *Parmelia sexatilis*, and *Parmelia caperata* showed largest DPPH radical scavenging activity (84.33%) which was similar or greater than the standard antioxidants, ascorbic acid (86.5%), BHA (79.78%) and a-tocopherol (63.99%). The scavenging activity was also good for the lichen *Everniastrum cirrhatum* (74.6 %) and *Parmelia pereoridisum* (54.22%). The other lichens showed slightly weaker DPPH◦ radical scavenging activities (below 50%) (Fig. 2)

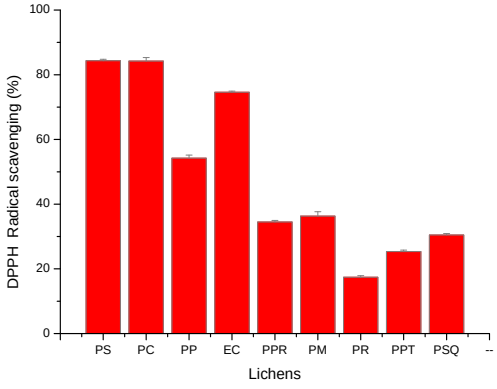

Figure 2 DPPH radical scavenging of the acetone extracts of the lichens; PS, Parmelia sexatilis; PC, Parmelia caperata; PP, Parmelia parletum; EC, Everniastrum cirrhatum; PP, Parmelia pereoridisum; PM, Parmoterma mesotropum; PR, Parmoterma reticulatum; PPT, Parmoterma perlatum; PSQ, Parmelia squarrosa; bar indicates the value of standard deviation.

4. Discussion

Lichens have been used in medicine by the ancient Chinese and Egyptians [15]. In the present study, in vitro antioxidant, and antimicrobial activities of ethanol extract from the lichens were examined. The tested lichen extracts showed strong antibacterial and antioxidant activity. The extracts of *Parmelia caperata, Parmelia parletum, Parmoterma* spp. had the strongest antibacterial activity among the tested species of lichens. The extract did not showed activity against fungi. Most of the lichen species were effective against *M. smegmatis* and *B. cereus* which in accordance with other studies, focused on the antimicrobial activities which have demonstrated that bacteria are more sensitive than the fungi [16]. Ranković *et al* [16] reported antimicrobial activity for the methanol extract of the lichens *Parmelia centrifuga*. Similarly, strong antimicrobial activity of *Parmelia sulcata* and *Parmelia reticulata* have been reported previously [17-18].

The lichen extracts have a strong antioxidant activity against various oxidative systems in vitro. During the study it was found that the tested extracts exhibited the highest radical scavenging activity with the greatest amount of phenolic content. The highest value of phenols was seen in the ethanol extract of *Parmelia caperata*, and *Everniastrum cirrhatum* which exhibited the strongest radical scavenging activity. Based on these results, it could be concluded that anti-oxidative nature of the extracts might depend on their phenolics. Phenolic components are potential antioxidants, free radical terminators [19-20]. These compounds are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step. This high potential of phenolic compounds to scavenge radicals such as singlet oxygen, superoxide and hydroxyl radicals may be explained by their phenolic hydroxyl groups [21].

In conclusion, it can be stated that tested lichen extracts have a strong antioxidant, and antibacterial activity in vitro. On the basis of these results, lichen appears to be good and safe natural antioxidant, antibacterial agents and also, could be of significance in human therapy and animal diseases. Further studies should be done

to search new compounds from lichens that exhibit strong antioxidant, and antimicrobial activity.

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